PI3K/mTOR signaling in mesothelioma patients treated with induction chemotherapy followed by extrapleural pneumonectomy

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Abstract: INTRODUCTION: The prognostic significance of activity biomarkers within the phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling pathway was assessed in two independent cohorts of malignant pleural mesothelioma (MPM) patients uniformly treated with a multimodal approach. We specifically assessed expression signatures in a unique set of pre- and postchemotherapy tumor samples. METHODS: Biomarker expression was assessed in samples of two independent cohorts of 107 (cohort 1) and 46 (cohort 2) MPM cases uniformly treated with platinum-based induction chemotherapy followed by extrapleural pneumonectomy from two different institutions, assembled on tissue microarrays. Expression levels of phosphatase and tensin homologue (PTEN), phospho-mTOR, and p-S6 in addition to marker of proliferation (Ki-67) and apoptosis (cleaved caspase-3) were evaluated by immunohistochemistry and correlated with overall survival (OAS) and progression-free survival (PFS). To assess PTEN genomic status, fluorescence in situ hybridization was performed. RESULTS: Survival analysis showed that high p-S6 and Ki-67 expression in samples of treatment naïve patients of cohort 1 was associated with shorter PFS ($p = 0.02$ and $p = 0.04$, respectively). High Ki-67 expression after chemotherapy remained associated with shorter PFS ($p = 0.03$) and OAS ($p = 0.02$). Paired comparison of marker expression in samples before and after induction chemotherapy of cohort 1 revealed that decreased cytoplasmic PTEN and increased phospho-mTOR expression was associated with a worse OAS ($p = 0.04$ and $p = 0.03$, respectively). CONCLUSIONS: These novel data reveal a prognostic significance of expression changes of PI3K/mTOR pathway components during induction chemotherapy if confirmed in other patient cohorts and support the growing evidence to target the PI3K/mTOR pathway in the treatment of MPM.

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Conclusions: These novel data reveal a prognostic significance of expression changes of PI3K/mTOR pathway components during induction chemotherapy if confirmed in other patient cohorts and support the growing evidence to target the PI3K/mTOR pathway in the treatment of MPM.

Key Words: Phosphatidylinositol 3-kinase/mammalian target of rapamycin, Phosphatase and tensin homologue, Ki-67, Mesothelioma, Multimodality treatment.

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Malignant pleural mesothelioma (MPM) is a rare and aggressive asbestos-related tumor,[1–3] with a median survival of 12 months or less without treatment.[4–6] Multimodal therapy including macroscopic complete resection is currently suggested for mesothelioma patients tailored to the patient's requirements and the stage of the disease.[7] One particular approach with promising survival data involves induction chemotherapy followed by extrapleural pneumonectomy (EPP).[8,9] Not all patients benefit from this treatment approach. Therefore, the identification of prognostic markers that may help in selecting patients remains a subject of key importance.[10]

In recent years, the phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mammalian target of rapamycin [mTOR]) pathway has been the focus of interest for identifying potential prognostic markers for cancer.[11–13] The PI3K/mTOR signaling pathway is activated by the binding of growth factors to cell surface receptor tyrosine kinases and as such is charged with integrating nutrient and energy homeostasis with mitogenic input.[13] Activation of the PI3K/mTOR pathway results in the regulation of cell growth, protein biosynthesis, and proliferation, all of which promote tumorigenesis.[12] Signaling of this pathway is however negatively regulated by the tumor suppressor phosphatase and tensin homologue (PTEN).[14] Accordingly, up-regulation of the PI3K/mTOR pathway, often through loss of PTEN function, has

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been detected in a variety of neoplasms including prostate,[15,16] breast,[17] and colorectal cancer[18] in addition to malignant melanoma[19] and has subsequently been targeted for therapeutic intervention.[12] Indeed, molecules targeting the PI3K/mTOR pathway have been developed and are currently being evaluated in various cancer clinical trials[20,21] including MPM (ClinicalTrials.gov Identifier: NCT01024946—Everolimus study).[12]

By using a tissue microarray (TMA)–based approach in a previous study, we observed a loss of PTEN protein in 62% of mainly untreated MPM patients.[22] A recent study by Cedrés et al.[23] confirmed that aberrant activation/expression in some constituent proteins of the PI3K/mTOR signaling cascade provides prognostic information in treatment-naïve patients with MPM. The present study therefore aimed to further elucidate the prognostic significance of the PI3K/mTOR signaling pathway in clinically annotated samples from two independent cohorts of MPM patients uniformly treated in a multimodal treatment concept. In this unique cohort of MPM patients, we specifically sought to identify the prognostic value of activity biomarkers of the PI3K/mTOR pathway in tumor samples collected before and after induction chemotherapy to define selection criteria for multimodality treatment.

PATIENTS AND METHODS

Patients

In this study, we included two independent cohorts (University Hospital Zurich, cohort 1, 1999–2009 [n = 107]; Toronto General Hospital, cohort 2, 2001–2011 [n = 46]) of MPM patients uniformly treated with induction chemotherapy followed by EPP as described previously.[8,24] The study was approved, and waivers of consent were granted by the Ethical Committee Zürich (StV 29–2009 and EK-ZH 2012-0094) and by an Institutional Ethical Review Board at the Toronto General Hospital (IRB No. 11-0167-T).

TMA Construction and Immunohistochemistry

A set of four TMAs with double prechemotherapy and quadruple postchemotherapy punches per patient was prepared with a custom-made, semiautomatic tissue arrayer (Beecher Instruments, Sun Prairie, WI), as previously described.[25] Recipient TMA blocks were sectioned and stained with hematoxylin and eosin for morphologic assessment. Technical factors leading to loss of patient material during TMA processing (i.e., sectioning and staining) may be reflected through a reduction in the patient numbers available for subsequent statistical analysis.

Deparaffinized 2-μm-thick TMA sections were automatically stained with BenchMark (Ventana, Tucson, AZ) using the iView diamino benzidine detection kit (Ventana). The primary antibodies were a mouse monoclonal antibody (Novocastra, Newcastle, United Kingdom) for PTEN (28H6) diluted at 1:200, rabbit polyclonal antibodies (Cell Signaling Technology, Danvers, MA) against phospho-S6 (p-S6, Ser240/244) at a 1:50 dilution, a rabbit monoclonal antibody (Cell Signaling Technology) for phospho-mTOR (p-mTOR, 49F9) at a 1:50 dilution, rabbit polyclonal antibodies (Abcam, Cambridge, United Kingdom) against cleaved (active) caspase-3 at a 1:50 dilution, and a rabbit monoclonal antibody (Ventana Roche, Tucson, AZ) for Ki-67 (30–9) at a 1:50 dilution.

Immunohistochemical evaluation of the TMAs was conducted by four independent observers (B.B., M.M., S.T., and L.F.) in a blinded manner and cross-checked by a senior pathologist (A.S.). The staining intensity was semiquantitatively scored 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). Furthermore, the percentage of cells having any positivity was proportionally scored 0 (0%), 0.1 (1%–9%), 0.5 (10%–49%), or 1.0 (50% and more) as previously described.[26] The H-score was obtained by multiplication of intensity with percentage staining (final range, 0 to 3, per core). The final H-score was determined by averaging the H-scores of all the cores from the same patient.

The proliferation and apoptotic indices were defined as the percentage of Ki-67 and cleaved caspase-3–positive tumor cells, respectively, in representative tumor areas or *hot spots*. Hot spots are areas of tumor that exhibit higher levels of proliferation or apoptosis than the rest of the tumor areas.[27,28] In each core, the amount of cells with nuclear Ki-67 immunostaining or which exhibited cellular cleaved caspase-3 expression among 100 tumor cells was carefully counted (i.e., the proliferation or apoptotic index = proliferation or apoptotic cell count/total cell count × 100%). The overall proliferation and apoptotic indices were calculated by summing the percentage of positive cells obtained from each core and dividing this value by the total number of cores assessed from each MPM patient.

Fluorescence In Situ Hybridization Probe Selection and Analysis

Fluorescence in situ hybridization (FISH) was conducted on a single 4-μm-thick postchemotherapy TMA section using the Vysis LSI PTEN (10q23)/CEP 10 Dual Color Probe (Abbott Molecular Inc., Abbott Park, IL) according to the manufacturer's description. Ten MPM patients from cohort 1 with high PTEN expression scores (H-score >2) and 10 patients from cohort 1 with low PTEN protein expression (H-score <1) were selected for PTEN analyses. FISH analysis was performed in a blinded manner using a section immediately adjacent to the first hematoxylin and eosin–stained slide to verify the presence of tumor. Samples were analyzed under a 100× oil immersion objective, using an Olympus BX-UCB fluorescence microscope equipped with appropriate filters, a charge-coupled device camera and the CytoVision FISH imaging and capturing software (Applied Imaging, San Jose, CA). Semiquantitative evaluation of the tests was independently performed by B.B. and cross-checked by a senior pathologist (A.S.). For each case, an attempt was made to analyze at least 100 well-delineated tumor cells. PTEN genomic status was assessed by comparing the number of (orange) PTEN probe signals with the number of (green) reference signals (CEP-10 probe specifically hybridizes alpha satellite sequences specific to centromere region of chromosome 10). Infiltrating lymphocytes (from within the tumor core biopsies) were used as a reference for determining cutoff values (i.e., mean percentage + three SDs[29]; see Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A499).

Statistical Analysis

The expression of the different markers before and/or after chemotherapy and the dynamic change during chemotherapy were assessed for their prognostical impact on overall survival (OAS) and progression-free survival (PFS) and were correlated to histological subtype, modified Response Evaluation Criteria in Solid Tumors (RECIST) criteria, pT or pN stage, and International Mesothelioma Interest Group (IMIG) stages. The cutoff values were defined based on the median score of the tumor samples undergoing similar treatment (i.e., prechemotherapy and postchemotherapy). Protein expression values above the median were classified as *High*, whereas protein expression values below the median were classified as *Low*. TMA spots with a lack of tumor tissue (sampling error), damaged tissue (heat or crush artifact), or a total lack of tissue at some array positions (*empty spots*) were excluded from the analysis, leading to different patient numbers in the subsequent analysis. Tumor response to treatment was evaluated based on modified RECIST criteria.[30] OAS was defined as time between application of the first cycle of chemotherapy and time point of death or last follow-up. PFS was defined as the time interval between start of the first cycle of chemotherapy and progression based on the first confirmed sign of disease being either evident clinically, histologically confirmed by biopsy or fine-needle aspiration or evident radiologically with increasing tumor masses on regular follow-up computed tomography scans. Median survival time was assessed by Kaplan–Meier curves, and the influence of the different prognostic factors was analyzed by log rank test. p values lower than 0.05 were considered statistically significant. All analyses were carried out using IBM SPSS Statistics, version 20 (SPSS Inc., Chicago, IL).

RESULTS

Patient Characteristics of the Two Study Cohorts

The characteristics of MPM patients in the two cohorts are summarized in Table 1. In both cohorts, the median age of MPM patients was 61, mostly males were affected (92% cohort 1 versus 80% cohort 2), and the most frequent tumor histotype was epithelioid (54% cohort 1 versus 78% cohort 2). Patients with IMIG stage III to IV were more frequent in cohort 2 (i.e., 64% cohort 1 versus 87% cohort 2). Prechemotherapy tissue samples were only available from cohort 1 (n = 87), whereas postchemotherapy samples were available in both cohort 1 (n = 106) and cohort 2 (n = 46). Paired samples with tissue before and after chemotherapy were available in 86 patients from cohort 1.

Expression Pattern of PTEN, p-S6, p-mTOR, Ki-67, and Cleaved Caspase-3 in MPM Tissue

PTEN protein was homogeneously expressed in both the cytoplasmic and nuclear compartments (Fig. 1*B* and *F*), whereas p-S6 (Fig. 1*C* and *G*) and p-mTOR (Fig. 1*D* and *H*) expression was mostly restricted to the cytoplasm of tumor cells. Notably, increased p-mTOR and p-S6 protein expression was detected within the tumor cells compared with the fibroblasts of the surrounding stroma (Fig. 1). Although Ki-67 expression was restricted to the nucleus, cleaved caspase-3 expression was located in both the nuclear and cytoplasmic cellular fractions.

A significant reduction in PTEN H-score (cytoplasmic, $p = 0.04$ and nuclear, $p < 0.0005$) was detected after chemotherapy in cohort 1 (n = 75) where biopsies were available before and after chemotherapy (Fig. 2). Similar levels of nuclear and cytoplasmic PTEN expression were observed after chemotherapy in cohort 2 (Supplementary Figure 1, Supplemental Digital Content 2, http://links.lww.com/JTO/A500). A significantly reduced expression profile was also observed for p-mTOR in tumor tissue collected after induction chemotherapy (n = 66; $p < 0.0005$), p-S6 (n = 72; $p < 0.0005$), and Ki-67 (n = 69; $p < 0.0005$) *in cohort 1*. As opposed to the other four markers, a significantly increased expression of cleaved caspase-3 was detected after chemotherapy (n = 74; $p = 0.03$) *in cohort 1* (Fig. 2).

TABLE 1. Patients Characteristics

	Cohort 1	Cohort 2	*p*
No. of patients	107	46	
Follow-up (months)	22 (3–121)	15 (4–115)	
Median age	61 (36–72)	61 (33–78)	0.9[a]
Sex			0.06[a]
Male	98 (92%)	37 (80%)	
Female	9 (8%)	9 (20%)	
Induction chemotherapy			
Cisplatin/gemcitabine	46 (43%)	6 (13%)	
Cisplatin/pemetrexed	58 (54%)	18 (39%)	
Other	3 (3%)	22 (48%)	
Adjuvant radiotherapy	62 (58%)	37 (80%)	
Histological features			0.02[a]
Epithelioid	57 (53%)	36 (78%)	
Sarcomatoid	5 (5%)	0	
Biphasic	45 (42%)	10 (22%)	
Tumor stage (IMIG)			0.003[a]
I	10 (9%)	1 (2%)	
II	29 (27%)	5 (11%)	
III	58 (55%)	27 (59%)	
IV	10 (9%)	13 (28%)	
pT stage			0.001
ypT 1	10 (9%)	1 (2%)	
ypT 2	40 (38%)	7 (15%)	
ypT 3	47 (44%)	25 (54%)	
ypT 4	10 (9%)	13 (29%)	
pN stage[b]			0.02
ypN 0	70 (66%)	20 (44%)	
ypN 1	14 (13%)	8 (17%)	
ypN 2	22 (21%)	17 (37%)	
ypN 3		1 (2%)	

[a]Significance was calculated with the Student's *t* test and the Fisher's exact test.
[b]Data were not available for all 107 patients.
IMIG = International Mesothelioma Interest Group.

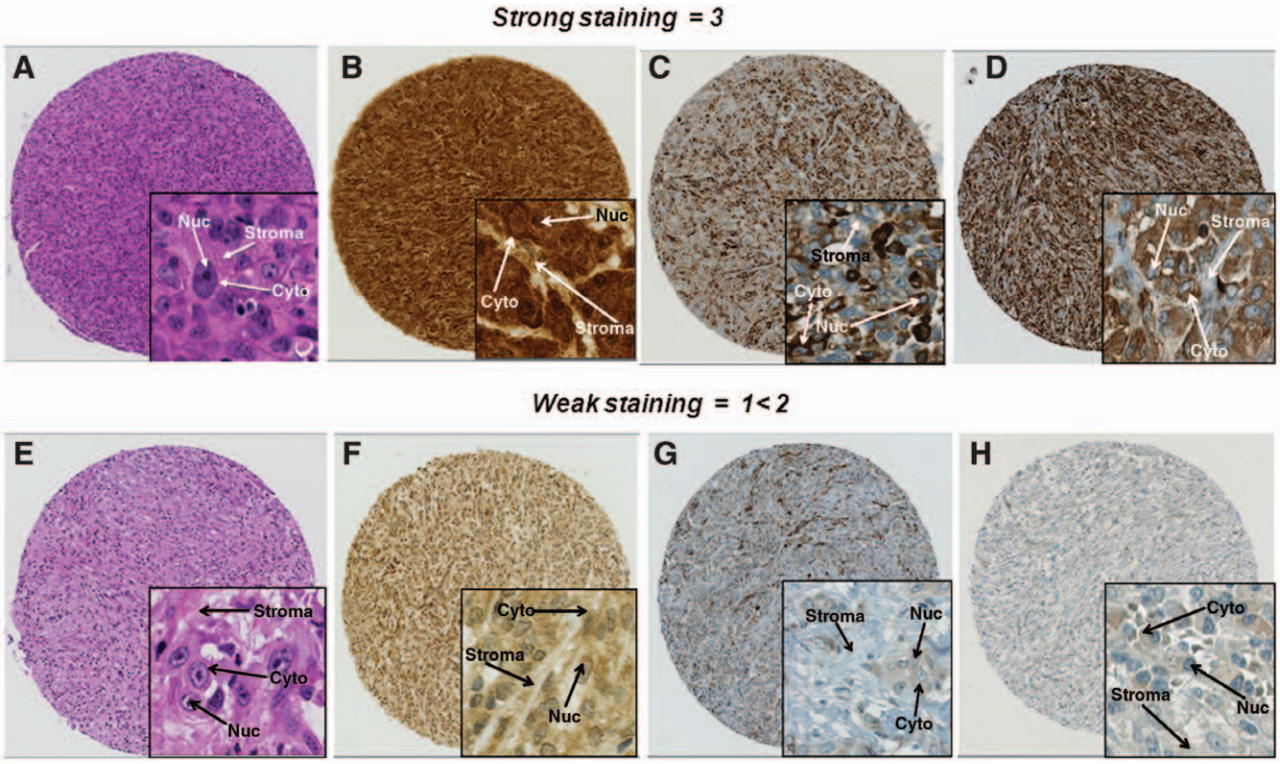

FIGURE 1. Immunohistochemical staining of PTEN, p-S6, and p-mTOR in MPM in an epithelioid subtype. Hematoxylin and eosin staining of respective MPM cores (*A* and *E*). Cytoplasmic and nuclear PTEN expression (*B*—strong and *F*—Weak). Cytoplasmic expression of p-S6 (*C*—strong and *G*—weak). Cytoplasmic expression of p-mTOR (*D*—strong and *H*—weak). Cyto = cytoplasm; Nuc = nucleus; PTEN, phosphatase and tensin homologue; p-mTOR, phospho-mammalian target of rapamycin; MPM, malignant pleural mesothelioma.

Prognostic Value of p-S6, PTEN, p-mTOR, Ki-67, and Cleaved Caspase-3 Expression

A significant association was found between the pre-chemotherapy expression level of p-S6 and Ki-67 with PFS ($p = 0.02$, n = 75 and $p = 0.04$, n = 74, respectively). Specifically, low expression of p-S6 was associated with longer PFS as opposed to high p-S6 expression (Fig. 3*A*). Similarly, low level of Ki-67 was found to be associated with a longer PFS as opposed to high Ki-67 levels (Fig. 3*B*).

Moreover, low expression levels of Ki-67 in postchemotherapy tissue was found to be associated with a longer PFS ($p = 0.03$, n = 101; Fig. 3*C*) and OAS ($p = 0.02$, n = 101; Fig. 3*D*, respectively).

In contrast, no significant association was detected between pre- and postchemotherapy biopsy expression levels of PTEN (nuclear and cytoplasmic), p-mTOR, and cellular cleaved caspase-3 with either OAS or PFS.

To assess the value of the dynamic changes observed during chemotherapy, we applied a paired sample analysis in which we compared the expression levels of the markers of interest (i.e., PTEN, p-mTOR, p-S6, Ki-67, and cleaved caspase-3) pre- and postchemotherapy of cohort 1. Survival analysis indicated that a reduction in cytoplasmic expression of PTEN was significantly associated with shorter OAS ($p = 0.04$, n = 75; Fig. 3*E*) but not PFS. Increased expression of p-mTOR had a significant prognostic impact on shorter OAS ($p = 0.03$, n = 66; Fig. 3*F*) but not on PFS. In contrast to PTEN and p-mTOR, no significant association between the relative expression of p-S6, caspase-3, and Ki-67 with OAS or PFS was detected.

There was no significant association between expression of the different markers in pre- or postchemotherapy expression and the clinico-pathological variables including histological subtype, modified RECIST criteria, pT or pN stage, and IMIG stages (data not shown). Similarly, the dynamic changes in marker expression did not correlate with any of the clinico-pathological variables.

PTEN FISH Assessment

To determine whether the reduction in PTEN protein expression observed in MPM patients after induction chemotherapy was owing to gene deletion, PTEN FISH was performed on a subset of these cases (i.e., MPM patients who either had high [H-score >2] or low [H-score <1] PTEN protein expression). None of the MPM cases evaluated showed PTEN hemizygous or homozygous deletion. Therefore, there was no correlation between PTEN protein expression level and PTEN FISH status (*data not shown*)

DISCUSSION

We have demonstrated a prognostic significance of the PI3K/mTOR signaling pathway for patients undergoing a multimodal treatment concept using a TMA-based approach in

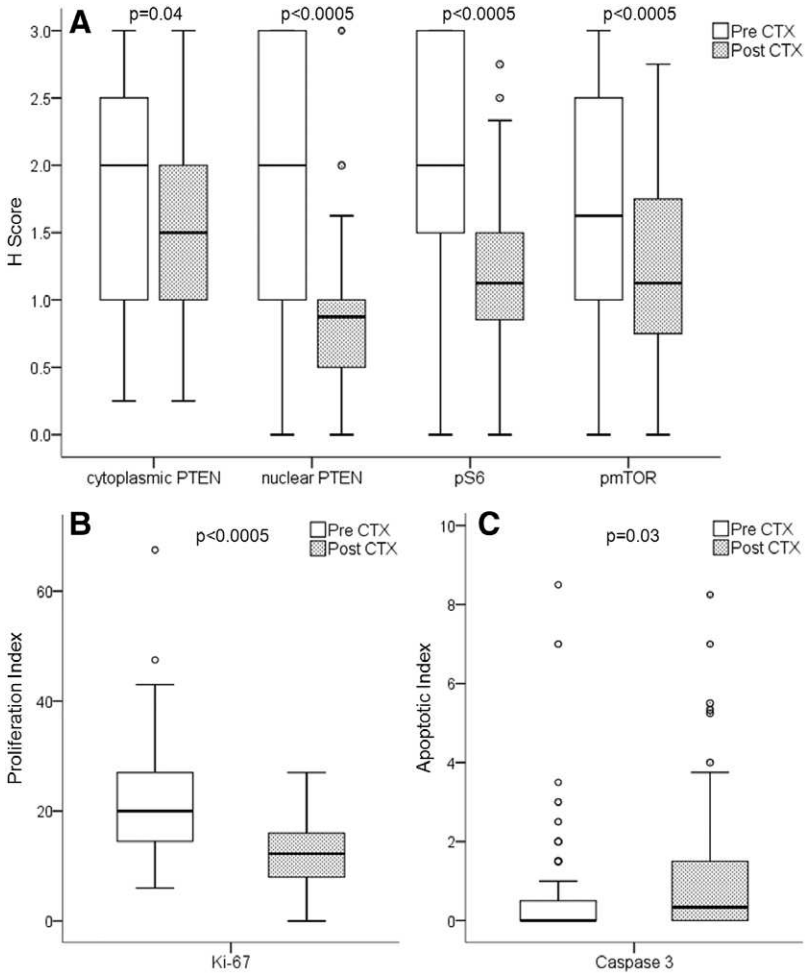

FIGURE 2. Box and whisker plots of expression levels of PTEN (cytoplasmic and nuclear), p-S6, p-mTOR, Ki-67, and cleaved caspase-3 in paired MPM data sets from pre- and postchemotherapy (CTX) biopsies. *A*, Expression levels (H-score) of cytoplasmic PTEN ($p = 0.04$), nuclear PTEN ($p < 0.0005$), p-S6 ($p < 0.0005$), and p-mTOR ($p < 0.0005$) were significantly reduced after CTX. *B*, Ki-67 proliferation index was significantly reduced after CTX ($p < 0.0005$). *C*, Apoptotic index by means of cleaved caspase-3–positive tumor fractions was significantly increased after CTX ($p = 0.03$). Significance was calculated with related-samples Wilcoxon signed-rank test. PTEN, phosphatase and tensin homologue; p-mTOR, phospho-mammalian target of rapamycin; MPM, malignant pleural mesothelioma.

two independent cohorts of MPM patients uniformly treated with induction chemotherapy followed by EPP. As a new finding, we observed that a decrease in PTEN and an increase in p-mTOR expression during induction chemotherapy were associated with shorter OAS; this seems to become particularly important on the long term after 12 months survival and longer. To our knowledge, this is the first study to investigate whether dynamic changes in biomarkers of activity within the PI3K/mTOR signaling pathway during induction chemotherapy influence MPM patients' survival.

We observed that PTEN levels were reduced after cisplatin-based induction chemotherapy both in the cytoplasm and the nucleus. Although the role of cytoplasmic PTEN to dampen the activity of the PI3K/mTOR pathway has been well documented,[14] the function of nuclear PTEN is gradually being unraveled.[31] Nuclear PTEN plays a role in DNA repair,[32] cell cycle arrest,[33] chromosome stability,[32] and tumor-suppressive activity.[34] The balance between the functions of these two cellular fractions of PTEN is an important factor in determining whether a cell remains benign or becomes neoplastic.[31] Although we observed a reduction in nuclear PTEN expression after induction chemotherapy, only the cytoplasmic expression of PTEN seemed to be associated with clinical outcome, stressing the role of PI3K/mTOR signaling in MPM progression.

The observed decrease in PTEN immunoreactivity after chemotherapy was unexpected. Although Krisman et al.[35] have reported genomic loss at the PTEN locus in MPM patients, in the current study we found no deletion, meaning that the reduction in the PTEN protein expression after chemotherapy is probably because of other mechanisms such as transcription regulation and protein destabilization.[36] This is not the first time that a decrease in biomarker expression has been observed after chemotherapy as similar changes have recently been reported in breast cancer.[37] We have previously reported loss of PTEN expression to be associated with shorter OAS.[22] In the present study, approximately 25% of MPM patients who underwent examination in both cohorts exhibited low PTEN expression (i.e., H-score 0–1.00). Agarwal et al.[38] have recently demonstrated in a set of 86 archival samples of untreated MPM patients that PTEN is absent in 26.7% of the patients when compared with expression in normal pleura. Cedrés et al.[23] reported 10% PTEN loss in the cytoplasmic fraction in archive biopsies of chemonaïve patients. Consistent with the aforementioned studies, we found no significant association between PTEN expression and survival in the present series of MPM patients. Potential explanations for these disparities may be differences in scoring methods and the MPM populations. Indeed, although in the present study

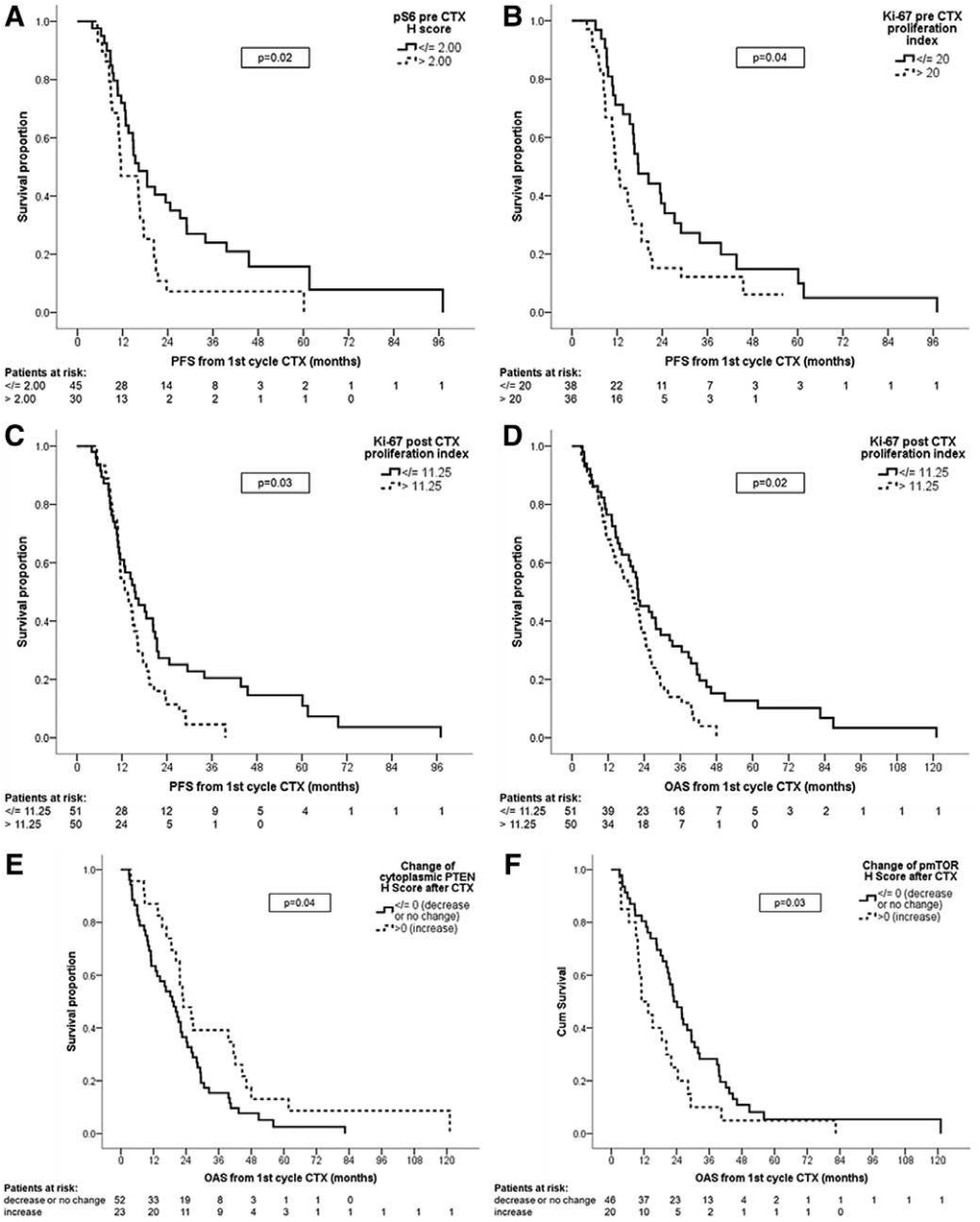

FIGURE 3. Kaplan–Meier curves according to PTEN, p-mTOR, p-S6, Ki-67, and cleaved caspase-3 expression levels in malignant pleural mesothelioma tissue samples. *A*, High p-S6 expression before chemotherapy (CTX) is associated with shorter PFS: patients with low to moderate p-S6 expression (median PFS = 16 months, 95% CI, 12–21, versus high p-S6 = 12 months, 95% CI, 5–18) (cohort 1, $n = 75$); Kaplan–Meier estimates of median time to progression (log rank test: $p = 0.02$). *B*, High Ki-67 proliferation index before chemotherapy is significantly associated with shorter PFS (low to moderate Ki-67 = 18 months, 95% CI, 13–23, against high Ki-67 = 12 months, 95% CI, 10–13) (cohort 1, $n = 74$); Kaplan–Meier estimates of median time to progression (log rank test: $p = 0.04$). *C*, Ki-67 proliferation index after chemotherapy is significantly associated with shorter PFS (low to moderate Ki-67 = 16 months, 95% CI, 10–21, against high Ki-67 = 14 months, 95% CI, 10–17) (cohort 1 $n = 101$); Kaplan–Meier estimates of median time to progression (log rank test: $p = 0.03$). *D*, High Ki-67 proliferation index after chemotherapy is significantly associated with shorter OAS (low to moderate nuclear Ki-67 = 22 months, 95% CI, 17–27 months, against high Ki-67 = 20 months, 95% CI, 15–25 months) (cohort 1 $n = 101$); Kaplan–Meier estimates of OAS (log rank test: $p = 0.02$). *E*, Reduced cytoplasmic PTEN expression is significantly associated with shorter OAS (decreased PTEN = 19 months, 95% CI, 13–24 months, against increased PTEN = 23 months, 95% CI, 16–30 months) (cohort 1 $n = 75$); Kaplan–Meier estimate for OAS (log rank test: $p = 0.04$). *F*, Increased cytoplasmic p-mTOR expression is significantly associated with shorter OAS (decreased p-mTOR = 23 months, 95% CI, 18–28, against increased p-mTOR = 11 months, 95% CI, 5–17 months) (cohort 1 $n = 66$); Kaplan–Meier estimate for OAS (log rank test: $p = 0.03$). PFS, progression-free survival; OAS, overall survival; PTEN, phosphatase and tensin homologue; CI, confidence interval.

biopsies were collected from MPM patients before or after induction chemotherapy, our original study was mainly composed of biopsies from MPM patients collected at autopsy (77%). Interestingly, PTEN expression has been reported to be reduced in advanced stages of prostate cancer[39] and in late-stage non–small-cell lung cancer compared with early-stage biopsies.[40] Even though PTEN expression was not significantly correlated with the IMIG stage in the MPM patients analyzed herein, we noted a trend toward a reduction in PTEN expression with increased IMIG stage (data not shown).

mTOR is a highly conserved serine/threonine kinase and a central regulator of cell growth and metabolism in eukaryotes.[13] Our data did not reveal any association between p-mTOR immunoreactivity in the pre- and postchemotherapy biopsies and survival when they were analyzed independently. Consistent with these findings, Cedrés et al.[23] have also reported no significant correlation between p-mTOR expression levels and survival in their cohort of chemotherapy-naïve MPM patients. Nevertheless, the observed decrease in p-mTOR expression after chemotherapy and its prognostic significance indicate that mTOR activation is modified by the treatment and that this change influences clinical outcome. Against this background, the administration of mTOR inhibitors to MPM patients who exhibit increased p-mTOR after chemotherapy treatment may be beneficial.

An integral downstream target of the PI3K/mTOR signaling pathway is the ribosomal protein S6, which is rapidly phosphorylated by the S6 kinase when cells are stimulated to grow or divide.[41] Indeed, we observed that increased expression of p-S6 in prechemotherapy tissue from MPM patients to be a prognostic factor influencing clinical outcome.[23] In this study, we confirm the association of high p-S6 expression with a shorter PFS as previously reported.[42] Interestingly, experimental models using human mesothelioma cell lines grown as spheroids have shown p-S6 to contribute to acquired apoptotic resistance.[43]

To gain insight into the histopathological changes underlying the reduced expression of the PI3K/mTOR activity biomarkers evaluated herein after induction chemotherapy, we decided to investigate apoptosis and proliferation using two bona fide markers, cleaved caspase-3 and Ki-67. Interestingly, we found an increased cleaved caspase-3 apoptotic index and a decreased Ki-67 proliferative index in postchemotherapy specimens. Consistent with four previous reports, we found a high proliferation index before chemotherapy to be associated with a worse prognosis.[44–47] Notably, our Ki-67 expression median scores were in close alignment with the MPM reports by Beer et al.[44] and Kadota et al.[45]

The changes observed herein suggest a link between decreased cell proliferation coupled with increased apoptosis and modification of PI3K/mTOR pathway components. This is further supported by previous data from our laboratory assessed in a smaller subset of patients, where significantly increased terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate-biotin nick end-labeling positivity was observed and where increased expression of the senescence marker plasminogen activator-inhibitor-1 in MPM tissue specimens collected after chemotherapy was associated with a worse clinical outcome.[26] However, our analyses did not reveal any significant correlation between expression of PTEN, p-mTOR, and p-S6 with either cleaved caspase-3 or Ki-67 indices, even though PI3K/mTOR signaling is a key regulator of cell survival and apoptosis.[48] This may be explained by different scoring methods applied (H-score approach for PI3K/mTOR activity markers versus hot spot labeling indices for Ki-67 and cleaved caspase-3). The percentage of positive tumor cells (labeling index) has been widely applied for the scoring of Ki-67 and apoptosis.[27,28,49] Nevertheless, this method was inappropriate for assessing PI3K/mTOR activity markers as they were expressed in cytoplasmic and nuclear fractions of the majority of tumor cells with different degree of intensity (Fig. 1). In addition, it is more than likely that other molecules deregulated in MPM could account for the regulation of proliferation and apoptosis besides PI3K/mTOR pathway. Future studies will be necessary to determine how PI3K/mTOR activity is modified by chemotherapy and its implication in the regulation of tumor cell survival. Nevertheless, these data suggest an ongoing response of tumor cells to chemotherapy confirming the reduced PI3K/mTOR signaling detected herein as a response mechanism to treatment which may ultimately impinge on patient survival.

There were limitations to the present study, including fewer numbers and less material of MPM patient samples prechemotherapy as opposed to postchemotherapy. This resulted in a reduction in the relevant number of paired samples which may have limited statistical power. In addition, because MPM is a particularly heterogeneous type of tumor, it is possible that even up to four cores per patient do not fully represent the nature of the individual tumor. Moreover, despite the fact that both cohorts underwent a similar treatment regimen and were observed during a comparable period of time (i.e., 10 years), one cannot ignore the bias that stems from the use of retrospective samples from different institutions, including the inability to control for all other factors (i.e., confounding variables), a limitation of all observational studies.

Thus, to summarize, pathway analysis using human MPM tumor TMAs may provide potentially significant information as to the selection process of MPM patients undergoing induction chemotherapy for mesothelioma surgery or rather molecular-targeted therapy. Furthermore, these biomarkers might be useful to assist with the designing of effective clinical trials. The novel data reported in the present study suggest that dynamic changes in PTEN and p-mTOR expression during induction chemotherapy represent prognostic factors for MPM patients' OAS. Multi-institutional efforts comprised larger cohorts will be necessary to validate our findings. Taken together, our data support a role for activation of PI3K/mTOR signaling in MPM, and support the growing evidence for the use of anticancer drugs that inhibit the PI3K/mTOR signaling pathway in the treatment of MPM.

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